Fractionation and identification of antioxidant peptides from an enzymatically hydrolysed *Palmaria palmata* protein isolate

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Abstract

Proteins derived from the macroalgal species *Palmaria palmata* have emerged as potential substrates for the generation of bioactive peptides. The aim of this study was to fractionate, identify and characterize antioxidant peptides from a *P. palmata* protein hydrolysate. The *P. palmata* protein hydrolysate generated with the food-grade proteolytic enzyme Corolase PP was sequentially fractionated using solid phase extraction and semi-preparative (SP) RP-HPLC. The most active SP-RP-HPLC peptide fraction (SP-RP-HPLC-30-F26) was analysed by ESI-MS/MS. Seventeen novel peptide sequences were identified in this fraction. Of the peptides selected for synthesis, Ser-Asp-Ile-Thr-Arg-Pro-Gly-Gly-Asn-Met, showed the highest oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) activity with values of 152.43 ± 2.73 and 21.23 ± 0.90 nmol TE/μmol peptide, respectively. The results presented herein indicate that *P. palmata* derived peptides may have potential applications as health enhancing ingredients and as food preservatives due to their antioxidant activity.

Keywords: antioxidant peptide; antioxidant; bioactive peptide; food protein hydrolysates; *Palmaria palmata;* peptide identification; simulated gastrointestinal digestion

1. Introduction

An increase in the production of reactive oxygen species (ROS) in conjunction with an overload on endogenous antioxidant defence systems (enzymatic and non-enzymatic) has been implicated as a possible causative factor for the initiation and/or progression of many chronic diseases. These include cardiovascular disease (CVD), cancer, diabetes mellitus, cataracts and neurodegenerative disorders (Lobo, Patil, Phatak, & Chandra, 2010; Valko et al., 2007). Free radicals and other ROS are generated as part of the normal metabolic processes in the body. Furthermore, the body can also be exposed to external sources of free radicals such as x-rays, ozone, cigarette smoking, air pollutants and industrial chemicals. Through evolution, aerobic organisms have developed highly advanced enzymatic and non-enzymatic antioxidant defence systems to counteract free radicals. However, at times when the body's metabolic oxidant/antioxidant equilibrium is altered (oxidative stress), excessive quantities of reactive radicals can cause cellular damage which, in turn, can initiate or promote many of the diseases listed above (Sarmadi & Ismail, 2010). With an increase in the prevalence of diseases where oxidative stress is believed to be a causative factor a need exists to identify natural antioxidant components with little or no adverse effects on health that may be used to combat oxidative damage within the body. Additionally, as there is increasing evidence linking synthetic antioxidants to negative health effects the food industry is looking for natural antioxidant components for application as alternative preservation agents in food products (Lobo et al., 2010; Shahidi & Zhong, 2005).

The beneficial effects of food-derived components, including peptides, in health promotion and disease prevention are being increasingly recognized. These short peptides, which in general range from 2-20 amino acids in size, are encrypted within

the primary sequence of the parent protein and become active when released during food processing (by enzymatic hydrolysis or fermentation) or during gastrointestinal transit (Harnedy & FitzGerald, 2012). A growing body of scientific literature indicates the potential of food protein-derived peptides as antioxidant agents to combat the deleterious effects associated with oxidative stress (Freitas et al., 2013; Power, Jakeman, & FitzGerald, 2013).

Palmaria palmata (*P. palmata*), a red macroalgal species containing relatively high levels of protein (35% (w/w)) is a rich source of bioactive proteinaceous components (Harnedy & FitzGerald, 2013b). More specifically, a number of *P. palmata* derived protein isolates, protein hydrolysates, peptide fractions and mycosporine-like amino acids have shown promising antioxidant activity *in vitro* (Beaulieu, Sirois, & Tamigneaux, 2016; Bondu et al., 2014; Harnedy & FitzGerald, 2013b; Harnedy, Soler-Vila, Edwards, & FitzGerald, 2014; Wang et al., 2010; Yuan, Bone, & Carrington, 2005; Yuan, Carrington, & Walsh, 2005; Yuan, Westcott, Hu, & Kitts, 2009). Bondu et al. (2014) and Beaulieu et al. (2016) have identified numerous peptides in *P. palmata* protein hydrolysate fractions showing high antioxidant activity. It was found that the majority of the peptides identified originated from ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO) and pigments such as allophycocyanin, phycoerythrin and phycocyanin. However, confirmatory studies with synthetic peptides were not performed to identify and confirm which of the peptides were potentially responsible for the observed antioxidant activity (Beaulieu et al., 2016; Bondu et al., 2014). The objective of the study herein was to fractionate, identify and characterize peptides with antioxidant activity from a *P. palmata* protein hydrolysate.

2. Materials and methods

2.1 Materials

Corolase® PP was provided by AB Enzymes (Darmstadt, Germany) and BC pepsin was provided by Biocatalysts (Cardiff, United Kingdom). HPLC grade water and acetonitrile (ACN) were obtained from VWR International (Dublin, Ireland). ESI low molecular mass tune mix was from Agilent Technologies (Cork, Ireland). The synthetic peptides Ala-Asn-Ala-Ala-Thr-Ile-Ile-Lys, Asn-Ala-Ala-Thr-Ile-Ile-Lys, Leu-Gly-Leu-Ser-Gly-Lys, Phe-Ile-Thr-Asp-Gly-Asn-Lys were obtained from Thermo Fisher Scientific (Ulm, Germany). The synthetic peptides Ser-Asp-Ile-Thr-Arg-Pro-Gly-Gly-Gln-Met, Asp-Asn-Ile-Gln-Gly-Ile-Thr-Lys-Pro-Ala, Leu-Ile-Thr-Gly-Ala, Leu-Ile-Thr-Gly-Ala-Ala, Leu-Ile-Thr-Gly-Ala-Ala-Gln-Ala, Val-Val-Pro-Thr, Gln-Ala-Arg-Gly-Ala-Ala-Gln-Ala, Leu-Thr-Leu-Ala-Pro-Lys, Leu-Thr-Ile-Ala-Pro-Lys, Ile-Thr-Leu-Ala-Pro-Lys and Ile-Thr-Ile-Ala-Pro-Lys were from GenScript (Piscataway, NJ). All other reagents were supplied by Sigma Chemical Company Ltd. (Wicklow, Ireland).

2.2 Sample preparation

P. palmata samples were collected at Spiddal Beach, Co. Galway, Ireland. Of the total quantity of sample utilized in the study 75% was collected in March 2013 and 25% in May 2013. Following harvesting, the seaweed was washed with filtered seawater to remove sand and epiphytes. The cleaned seaweed was freeze-dried, mixed and pulverized with a Cyclotec™ Mill (1 mm screen, FOSS Tecator AB, Hoganas, Sweden) and stored in an air tight container at room temperature.

2.3 Generation of *P. palmata* protein isolate and associated hydrolysate

Crude aqueous soluble protein isolates were extracted using the method previously described (Harnedy & FitzGerald, 2013b). In brief, 845g of the milled freeze-dried seaweed was suspended in 16.9 L de-ionised H_2O (1:20 (w/v)) and mixed gently for 3 h at room temperature. The seaweed suspension was centrifuged at 4,190 x g (Sorvall RC6 Plus, Fisher Scientific, Dublin, Ireland) for 15 min at room temperature and the supernatant retained. Aqueous soluble proteins in the supernatant were precipitated by isoelectric precipitation at pH 3.5 using 1.0 M HCl. The precipitated protein was resuspended in dH_20 to a protein concentration of ~ 2.5% (w/v). The protein content of the crude aqueous soluble protein isolates was determined using the modified Lowry protein quantification method previously described (Harnedy & FitzGerald, 2013a). Bovine serum albumin was used to prepare a protein standard curve in the range 0-200 µg/ml.

The aqueous protein hydrolysate was generated with Corolase PP. A 2% (w/v) protein solution was adjusted to pH 7.0 with 1M NaOH and preheated to 50 °C prior to addition of the enzyme at an enzyme:substrate (E:S) ratio of 1% (w/w). The hydrolysis reaction was maintained at pH 7.0 using a pH-stat (842 Titrando, Metrohm, Switzerland). The enzyme was inactivated after 240 min by thermal inactivation at 90 ^oC for 20 min and the hydrolysate sample was subsequently freeze-dried (FreeZone 18L, Labconco, MO, USA) and stored at -20 ^oC.

2.4 Fractionation of the aqueous *P. palmata* protein hydrolysate

The aqueous *P. palmata* protein hydrolysate was fractionated using solid phase extraction (SPE) followed by semi-preparative reverse phase-high performance liquid chromatography (SP-RP-HPLC) as previously described (Harnedy, O'Keeffe, & FitzGerald, 2015). A 1% (w/v) protein hydrolysate (in HPLC-grade H_2O) solution was

applied to a pre-activated SPE column (Strata-X 5g/60mL C18, Phenomenex, Cheshire, UK). Material that passed through the column was termed 'unbound material'. Peptide fractions were sequentially eluted from the column using 10 ml HPLC grade H_2O and 10, 20, 25, 30, 40 and 60% (v/v) ACN. This was repeated 10 times and the individual fractions from each of the runs were pooled and dried using a solvent evaporator (Genevac, EZ-2 Plus, Genevac Ltd., Ipswich, UK). SP-RP-HPLC was utilized to further fractionate SPE-30% (the SPE peptide fraction that eluted at 30% (v/v) ACN). The dry SPE fractions were reconstituted at a concentration of 15 mg/ml (dw) in 0.1% (v/v) formic acid (FA) in HPLC grade H_2O (Mobile Phase A) and a 500 µL aliquot was injected onto a column (Jupiter C18, 250 × 15 mm ID, 10 mm particle size, 300Å pore size) fitted with a C18 guard column (10 × 10 mm ID, Phenomenex Cheshire, UK) which was connected to a Waters SP-HPLC (Waters, Dublin, Ireland). Mobile phase B consisted of 80% (v/v) ACN containing 0.1% (v/v) FA. The flow rate was set at 5 ml/min and the peptides were separated using the following gradient: 0–10 min: 0% B; 10–15 min: 0-18% B; 15-76 min: 18-60% B; 76-81 min: 60-100% B; 81-86 min: 100% B; 86-91 min: 100-0% B; 91–96 min 0% B. The absorbance of the eluent was monitored at 214 nm. Fractions eluting from 20 – 60 min were collected every minute using a fraction collector (Waters, Dublin, Ireland). Individual 1 min fractions from multiple runs were pooled and dried using a solvent evaporator.

2.5 Identification of peptides by UPLC-ESI-MS/MS

The separation and identification of peptides in the most active semi-preparative RP-HPLC fraction, SP-RP-HPLC-30-F26, was performed as previously described (Harnedy et al., 2015; O'Keeffe & FitzGerald, 2015). In brief, a 7 µl aliquot of a 1 mg/ml solution of the fraction prepared in mobile phase A (0.1% FA in MS grade H_2O),

was injected onto an ACQUITY BEH 300 C18 RP column (2.1 x 50 mm, 1.7 µm; Waters, Dublin, Ireland) connected to an ACQUITY UPLC (Waters, Dublin, Ireland). Mobile phase B was 0.1% FA in 80% (v/v) ACN. Peptides were separated as follows: 0–1 min: 0% B; 1-2 min: 0-6% B; 2-52 min: 6-25% B; 52-53 min: 25-100% B; 53-58 min: 100-50% B; 58-63 min: 50-0% B; 63-68 min: 0% B at a flow rate of 0.2 ml/min and monitored at 214 and 280 nm. MS analysis was performed on a micrOTOF Q II mass spectrometer (Bruker Daltonics, Bremen, Germany) in positive ion mode and full scans were carried out for Auto MS/MS between 100-2500 m/z. The MS/MS settings were as follows: capillary voltage 4500V; nebuliser pressure 1.8 Bar; dry gas flow 8 L/min; dry gas temp, 220 ˚C; collision energy, 10 eV; collision cell RF 350 Vpp, transfer time 120 µs. PEAKS Studio 6.0 software (Bioinformatics Solutions Inc, Waterloo, Canada) was used to search all MS/MS spectra on the Mascot search engine against the SwissProt Eukaryota database. Homology analysis to macro- and micro-algal protein sequences in an in-house database was used to generate *de novo* sequences and all peptide sequences determined were manually confirmed using the Annotate tool of Data Analysis software (Bruker Daltonics).

All peptides showing homology to macroalgal proteins were chemically synthesized. The synthetic peptide, Ser-Asp-Ile-Thr-Arg-Pro-Gly-Gly-Asn-Met, and the corresponding peptide fractions generated following simulated gastrointestinal digestion (SGID,7µl of 0.005 mg ml^{-1} in mobile phase A (0.1% formic acid (FA) in MS-grade H_2O) were separated and identified using an Acquity UPLC (Waters, Dublin, Ireland) coupled to an Impact HD Ultra high resolution (UHR) Q-TOF (Bruker Daltonics, Bremen, Germany). The MS/MS methods for long and short peptides were as previously published in O'Keeffe and FitzGerald (2015) and O'Keeffe, Norris, Alashi, Aluko, and FitzGerald (2017). The MS was calibrated with ESI tune mix

(Agilent) for the long peptide method and with sodium formate for the short peptide method. Masses of peptides expected to be generated following *in silico* peptic and Corolase PP digestion (BIOPEP: http://www.uwm.edu.pl/biochemia/index.php/en/biopep: using 'pepsin pH1.3' enzyme selection for *in silico* peptic digestion and 'elastase (Type 1), chymotrypsin C, trypsin and combinations thereof' for *in silico* Corolase PP digestion) were inserted on an include list in OTOF Control (Bruker Daltonics) such that these masses were preferentially fragmented in the MS.

2.6 Simulated gastrointestinal digestion (SGID)

The synthetic peptide showing high antioxidant activity was subjected to SGID by the method described previously (Harnedy et al., 2015; Walsh et al., 2004). In brief, peptides at a concentration of 20 mM were incubated at 37 ˚C and pH 2 for 90 min with pepsin at an enzyme:substrate ratio of 2.5% (w/w). A sub-sample representing simulated gastric digestion was removed and heat inactivated at 90 ˚C for 20 min. The remainder of the reaction mixture was adjusted to pH 7.5 using 1M NaOH and incubated for a further 150 min at 37 ˚C with Corolase PP (enzyme:substrate ratio of 1% (w/w)). This simulated gastrointestinal digestion sample was inactivated as described above. All samples were stored at 4 ˚C prior to antioxidant activity assessment.

2.7 Antioxidant activity

Antioxidant activity was determined *in vitro* using the oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays as previously described (Harnedy & FitzGerald, 2013b). The ORAC and FRAP values were

expressed as μmol of Trolox equivalents per gram dry weight (μmol TE/g dw) for the unhydrolysed protein extract, the protein hydrolysate, the SPE- and the SP-RP-HPLC-fractions. The ORAC and FRAP antioxidant activities of the synthetic peptides were expressed as nmol TE/μmol peptide. All results were presented as mean ± standard deviation of independent triplicate analyses (n=3).

2.8 Statistical analysis

All statistical analysis was performed using the statistical software program SPSS (Version 22, IBM Inc., Chicago, IL, USA). Statistical significance at a level of $p<0.05$ was determined using one-way analysis of variance (ANOVA) followed by Tukey's and Games–Howell post-hoc tests, where applicable.

3. Results and Discussion

3.1 Bioassay-guided fractionation of antioxidant peptides from the *P. palmata* protein hydrolysate

In a previous study we have shown that a *P. palmata* protein hydrolysate generated from the aqueous soluble protein fraction with the proteolytic enzyme preparation Corolase PP displayed high ORAC and FRAP antioxidant activity (Harnedy & FitzGerald, 2013b). The hydrolysate generated in a similar manner in this study from a newly harvested macroalgal sample with an extent of hydrolysis of 29.58 mg amino group g^{-1} protein showed similar ORAC (Table 1, 483.68 ± 31.09 μmol TE/g dw) and FRAP (Table 1, 25.29 ± 0.66 μmol TE/g dw) values to those previously reported which were 467.54 ± 26.73 and 21.59 ± 0.55 μmol TE/g dw for ORAC and FRAP antioxidant activity, respectively (Harnedy & FitzGerald, 2013b). While the antioxidant activity of *P. palmata* protein hydrolysates generated with a range of proteolytic enzymes have

been reported, the identity of the peptide sequences potentially responsible for the observed antioxidant activity in these hydrolysates have not been identified (Beaulieu et al., 2016; Bondu et al., 2014; Harnedy & FitzGerald, 2013b; Harnedy et al., 2014; Wang et al., 2010).

In this study, peptides were separated based on their hydrophobic properties using RP C18 SPE cartridges followed by semi-preparative RP-HPLC. As shown in Table 1, the antioxidant activities of peptide fractions eluted with ACN were significantly higher than the unbound peptide fraction and the peptide fraction eluted with H_2O. Furthermore, the ORAC and FRAP values recorded for the hydrophobic fractions, with the exception of the peptide fraction eluted with 20% (v/v) ACN, were significantly higher than those observed for the unfractionated hydrolysate. The highest ORAC activity was recorded with the peptide fractions eluted with 25, 30, 40% (v/v) ACN (Table 1), while the highest FRAP activity was observed with the peptide fractions eluted with 30 and 40% (v/v) ACN (Table 1). The SPE fraction eluted with 30% (v/v) ACN was selected for further fractionation as insufficient material was recovered following elution with 40% (v/v) ACN.

The SP-RP-HPLC profile of SPE-30% and the associated ORAC activity of the lyophilized RP fractions taken therefrom between 20 and 60 min (F20-F60) are shown in Fig 1. Fraction 26, the peptide fraction eluting at 26 min displayed significantly higher ORAC activity than all other fractions (Fig 1, 4380.75 ± 66.44 µmol TE/g dw). With the exception of F26, the quantity of material recovered following semi-preparative RP-HPLC fractionation was too low to perform FRAP analysis. A FRAP value of 51.86 ± 1.85 µmol TE/g dw was obtained for F26. This value was lower than that obtained for SPE-30%, the fraction from which it arose (Table 1, 110.35 ± 2.63 µmol TE/g dw). It is possible that one of the other SP-RP-HPLC fractions may have

higher FRAP activity or that the high FRAP activity seen in SPE-30% was a result of a mixture of peptides from different fractions mediating a synergistic effect.

3.2 Identification of *P. palmata* derived antioxidant peptides

Mass spectrometry (MS) analysis was performed on SP-RP-HPLC-30-F26 in an attempt to identify the peptides potentially associated with the observed antioxidant activity. This was performed using a database (Swiss-Prot) driven search approach and by *de novo* sequencing. Table 2 provides a list of all the peptides identified in SP-RP-HPLC-30-F26 and Fig. 2 is a representative mass spectrum for Ser-Asp-Ile-Thr-Arg-Pro-Gly-Gly-Gln-Met. Eleven of the seventeen peptide sequences identified were homologous to available macroalgal protein sequences. These include Phe-Ile-Thr-Asp-Gly-Asn-Lys, Asn-Ala-Ala-Thr-Ile-Ile-Lys, Ala-Asn-Ala-Ala-Thr-Ile-Ile-Lys, Ser-Asp-Ile-Thr-Arg-Pro-Gly-Gly-Gln-Met, Asp-Asn-Ile-Gln-Gly-Ile-Thr-Lys-Pro-Ala, Leu-Ile-Thr-Gly-Ala, Leu-Ile-Thr-Gly-Ala-Ala, Leu-Ile-Thr-Gly-Ala-Ala-Gln-Ala, Leu-Gly-Leu-Ser-Gly-Lys, Leu/Ile-Thr-Leu/Ile-Ala-Pro-Lys and Val-Val-Pro-Thr. The majority of the peptides identified in this fraction originated from pigment proteins such as allophycocyanin, phycoerythrin and phycocyanin. This was also seen in the studies performed by Bondu et al. (2014) and Beaulieu et al. (2016) where the highest number of peptides identified in their *P. palmata* hydrolysate subfractions showing high antioxidant activity were from pigment proteins. In addition, Bondu et al. (2014) and Beaulieu et al. Beaulieu et al. (2016) reported that a large number of the peptides identified in the active sub-fractions were from the sequence of RuBisCO, an enzyme involved in photosynthesis. In contrast, only one peptide identified in this study originated from this metabolic plant enzyme.

Of the eleven peptides originating from macroalgal proteins, six were homologous to protein sequences from Rhodophyta species that have been confirmed on a protein level (Asn-Ala-Ala-Thr-Ile-Ile-Lys, Ala-Asn-Ala-Ala-Thr-Ile-Ile-Lys**,** Leu-Ile-Thr-Gly-Ala, Leu-Ile-Thr-Gly-Ala-Ala, Leu-Ile-Thr-Gly-Ala-Ala-Gln-Ala and Leu-Gly-Leu-Ser-Gly-Lys) and one peptide (Asp-Asn-Ile-Gln-Gly-Ile-Thr-Lys-Pro-Ala) where the protein sequence has been confirmed at transcription level. The latter peptide is homologous to G*riffithsia japonica* Histone H4 protein (Q7XY85). Four of the eleven peptide sequences are homologous to red macroalgal protein sequences that have only been inferred from homology. In this instance the protein sequence can be predicted because clear orthologs exist in closely related species.

Phe-Ile-Thr-Asp-Gly-Asn-Lys and Ser-Asp-Ile-Thr-Arg-Pro-Gly-Gly-Gln-Met are homologous to *P. palmata* phycoerythrin beta chain (F2ZAL7) and allophycocyanin beta chain (M1VJV1), respectively. With the exception of the Thr residue, all amino acid residues in Phe-Ile-Thr-Asp-Gly-Asn-Lys are highly conserved in the phycoerythrin beta chain of other red macroalgal species, *Porphyridium purpureum* (P11393), *Porphyridium sordidum* (P29948), *Rhodella violacea* (Q02037), *Polysiphonia urceolata* (P84862), *Gracilaria chilensis* (Q7SIF), *Griffithsia monilis* (O36004) and *Polysiphonia boldii* (Q01922), all of which have been confirmed on a protein level. The Thr residue is replaced with Ala, Asn, or Ser in these macroalgal species and it may be possible that the sequence in *P. palmata* is Phe-Ile-Thr-Asp-Gly-Asn-Lys and that a one codon difference at a genetic level translates to Thr as opposed to Ala, Asn or Ser. The peptide sequence Ser-Asp-Ile-Thr-Arg-Pro-Gly-Gly-Gln-Met is conserved as Ser-Asp-Ile-Thr-Arg-Pro-Gly-Gly-Asn-Met in *Porphyra yezoensis* (P59857) and *Galdieria sulphuraria* (P00319) allophycocyanin beta chain. Both sequences are evident on a protein level. Again, it may be possible that a one codon

difference at a genetic level translates to a Gln in *P. palmata* allophycocyanin beta chain as opposed to Asn. The peptide sequence Leu/Ile-Thr-Leu/Ile-Ala-Pro-Lys is conserved as Leu-Thr-Ile-Ala-Pro-Lys in two red macroalgal transaldolase protein sequences. However, *Galdieria sulphuraria* (M2Y2Z5) and *Chondrus crispus* (R7QKU4) transaldolase sequences are only inferred from homology. Therefore, because the protein sequences have not been confirmed on a protein level and as no experimental evidence exists on a transcription level it is difficult to confirm whether the first and third residues are Ile or Leu. The tetra peptide Val-Val-Pro-Thr is found in a number of red macroalgal protein sequences. However, all of these sequences are only inferred from homology.

To the best of our knowledge, none of peptides identified in this study, with the exception of Leu-Gly-Leu-Ser-Gly-Lys have previously been reported in the literature. The pentapeptide Leu-Gly-Leu-Ser-Gly which is similar to Leu-Gly-Leu-Ser-Gly-Lys was previously identified in a *P. palmata* derived SP-RP-HPLC peptide fraction showing high DPP-IV inhibitory activity (Harnedy et al., 2015). Furthermore, a similar peptide, Ser-Asp-Ile-Thr-Arg-Pro-Gly-Gly-Asn-Met-Tyr, to the peptide identified in this study (Ser-Asp-Ile-Thr-Arg-Pro-Gly-Gly-Gln-Met) has previously been identified in a fraction showing high antioxidant activity (Beaulieu et al., 2016). However, the sequence of this peptide was identified from the *P. palmata* allophycocyanin beta chain sequence which is only inferred from homology. In the current study the penultimate C-terminal residue was identified as Gln as opposed to Asn (Fig 2) which suggests that Asn is not conserved at this position in the allophycocyanin beta chain sequence of all Rhodophyta species.

3.3 Antioxidant activity of selected synthetic peptides.

In an attempt to identify the peptide(s) potentially responsible for the observed antioxidant activity in the active SP-RP-HPLC peptide fraction, all peptides showing homology to macroalgal proteins were chemically synthesised and assessed for peroxyl radical scavenging and ferric ion reducing activity. As shown in Table 3 the peptide, Ser-Asp-Ile-Thr-Arg-Pro-Gly-Gly-Gln-Met, displayed significantly higher ORAC and FRAP activity than all other peptides with values of 152.43 ± 2.73 and 21.23 ± 0.90 nmol TE/μmol peptide obtained, respectively.

The structure-activity relationship of antioxidant peptides has not yet been fully elucidated. However, the length of the peptide (3-16 amino acids), the presence of aromatic amino acids such as Tyr, His, Trp, and Phe and hydrophobic amino acids including Val, Leu, Ile, Pro, Ala, Met and Gly within a peptide, along with the structure at the C- and N- terminal residue is believed to influence antioxidant activity (Wang, Zhao, & Wang, 2013; Zou, He, Li, Tang, & Xia, 2016). Therefore, it is possible that the antioxidant activity observed with Ser-Asp-Ile-Thr-Arg-Pro-Gly-Gly-Gln-Met may be associated with the presence of Ile, Pro, Gly and Met residues within its sequence. According to structure-activity relationship studies performed with quantitative structure–activity relationship (QSAR) modelling the presence of bulky hydrophobic amino acids at the C-terminal region, in particular at position C3, is believed to influence the peroxyl radical scavenging activity of peptides (Li & Li, 2013). However, Asn-Ala-Ala-Thr-Ile-Ile-Lys and Ala-Asn-Ala-Ala-Thr-Ile-Ile-Lys which contain Ile at C3, were shown to have low activity (Table 3). It is possible that the absence of other key residues, i.e., aromatic amino acids, within the sequence may have contributed to the low antioxidant potency observed. The result of a chemometric multivariate analysis study identified amino acid composition as a significant contributor to FRAP activity and in particular it was found that sulphur-containing amino acids such as Cys

and Met are the most potent at reducing the Fe^{3+}-2,4,6-tripyridyl-*S*-triazine complex (Udenigwe & Aluko, 2011). Therefore, it may be possible that the high FRAP value observed with Ser-Asp-Ile-Thr-Arg-Pro-Gly-Gly-Gln-Met may be due to the Met residue. It was proposed that the positive contribution of Met to FRAP antioxidant activity is due to its sulphur group, which is prone to oxidation by the reactive species (Udenigwe & Aluko, 2011). Other amino acid residues such as Cys, Glu, Gln, Asp, Asn, Thr and Gly are also believed to contribute to ferric reducing power while Lys was shown to have a highly negative effect on FRAP activity (Udenigwe & Aluko, 2011). Interestingly, nine of the thirteen peptides with FRAP activity <1.6 nmol TE/µmol peptide contain a Lys residue with eight of these found at the peptide's C terminus. In addition, it may be possible that the 3-D structure of a peptide could impact on antioxidant activity; however, this was not investigated as part of this study. There is limited information in relation to the FRAP activity of synthetic peptides, however, the ORAC value recorded for Ser-Asp-Ile-Thr-Arg-Pro-Gly-Gly-Gln-Met, the peptide showing highest activity (Table 3, 152.43 ± 2.73 nmol TE/µmol peptide (0.152 ± 0.003 µmol TE/µmol peptide) was lower than the ORAC values reported for antioxidant peptides identified from milk and egg white proteins (0.306-0.799 µmol TE/µmol peptide, Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005; López-Expósito, Quirós, Amigo, & Reci, 2007; Nimalaratne, Bandara, & Wu, 2015). This activity was significantly lower than antioxidant peptides mined from β-lactoglobulin, κ-casein, ovotransferrin, peanut, walnut and egg white protein where ORAC values in the range of 1.240-15.470 µmol TE/µmol peptide were reported (Chen et al., 2015; Dávalos, Miguel, Bartolomé, & López-Fandiño, 2004; Hernández-Ledesma et al., 2005; López-Expósito et al., 2007; Nimalaratne et al., 2015; Power, Fernández, Norris, Riera, & FitzGerald, 2014; Shen, Chahal, Majumder, You, & Wu, 2010; Zheng et al., 2012).

However, to the best of our knowledge, this is the first report of a peptide exhibiting both peroxyl radical scavenging and ferric ion reducing activity.

In vitro digestion studies are often employed to assess the potential stability of a bioactive peptide as it passes through the gastrointestinal tract. Therefore, Ser-Asp-Ile-Thr-Arg-Pro-Gly-Gly-Gln-Met which exhibited high antioxidant activity was subjected to SGID. As shown in Table 4 no significant difference in both ORAC and FRAP activity was observed with Ser-Asp-Ile-Thr-Arg-Pro-Gly-Gly-Gln-Met during simulated gastric digestion. This was confirmed by MS analysis where Ser-Asp-Ile-Thr-Arg-Pro-Gly-Gly-Gln-Met remained intact during incubation with pepsin (Table 4). However, significantly lower ORAC (125.35 ± 2.04 nmol TE/μmol peptide) and FRAP (2.18 ± 0.07 nmol TE/μmol peptide) activity was observed following simulated intestinal digestion. Six peptides, Ser-Asp-Ile-Thr-Arg-Pro-Gly-Gly-Gln, Ser-Asp-Ile-Thr-Arg-Pro-Gly-Gly, Ser-Asp-Ile-Thr, Pro-Gly-Gly-Gln, Ser-Asp, and Gln-Met and Met were generated during simulated intestinal digestion (Table 4). While information in relation to the structure-activity relationship of peptides exhibiting ORAC activity is limited, there is little or no information with regard to the effect of peptide structure on FRAP activity. Therefore, it is difficult to predict which amino acid residues or combinations of amino acid residues in the peptide are associated with the observed activity without performing confirmation studies on the peptide fragments/amino acids generated by the action of Corolase PP. However, overall a more pronounced negative effect on FRAP activity was observed following gastrointestinal digestion of the peptide than that observed with ORAC activity.

4. Conclusions

In the present study a novel decapeptide, Ser-Asp-Ile-Thr-Arg-Pro-Gly-Gly-Gln-Met, with antioxidant activity was identified from an aqueous *P. palmata* protein hydrolysate. The results presented herein indicate that a *P. palmata* derived peptide or protein hydrolysate, which contains a complex mixture of peptides, may be a good source of antioxidant peptides for food and nutraceutical applications. However, it is necessary to perform further studies to investigate the ability of the protein hydrolysate/peptides to exhibit antioxidant activity in lipophilic systems, which is important both in a biological and food context. Furthermore, if the macroalgal-derived protein hydrolysate/peptide is to be utilized as an agent to combat oxidative stress, measures would need to be taken to protect the active components during intestinal digestion. Ultimately, a greater understanding of the biological fate of antioxidant peptides, where the efficacy of such peptides is confirmed in acute and long term *in vivo* studies, is required.

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449

450 **Conflict of Interest:** The authors declare that they have no conflict of interest.

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452 **References**

453 Beaulieu, L., Sirois, M., & Tamigneaux, E. (2016). Evaluation of the in vitro
454 biological activity of protein hydrolysates of the edible red alga, *Palmaria*
455 *palmata* (dulse) harvested from the Gaspe coast and cultivated in tanks.
456 *Journal of Applied Phycology, 28* (5), 3101-3115.
457 Bondu, S., Bonnet, C., Gaubert, J., Deslandes, É., Turgeon, S., & Beaulieu, L. (2014).
458 Bioassay-guided fractionation approach for determination of protein
459 precursors of proteolytic bioactive metabolites from macroalgae. *Journal of*
460 *Applied Phycology, 27* (5), 2059–2074.
461 Chen, H., Zhao, M., Lin, L., Wang, J., Sun-Waterhouse, D., Donga, Y., Zhuang, M.,
462 & Su, G. (2015). Identification of antioxidative peptides from defatted walnut
463 meal hydrolysate with potential for improving learning and memory. *Food*
464 *Research International, 78*, 216–223.
465 Dávalos, A., Miguel, M., Bartolomé, B., & López-Fandiño, R. (2004). Antioxidant
466 activity of peptides derived from egg white proteins by enzymatic hydrolysis.
467 *Journal of Food Protection, 67* (9), 1939-1944.
468 Freitas, A. C., Andrade, J. C., Silva, F. M., Rocha-Santos, T. A. P., Duarte, A. C., &
469 Gomes, A. M. (2013). Antioxidative peptides: trends and perspectives for
470 future research. *Current Medicinal Chemistry, 20*, 4575-4594.
471 Harnedy, P. A., & FitzGerald, R. J. (2012). Bioactive peptides from marine
472 processing waste and shellfish: A review. *Journal of Functional Foods, 4* (1),
473 6-24.
474 Harnedy, P. A., & FitzGerald, R. J. (2013a). Extraction of protein from the macroalga
475 *Palmaria palmata*. *LWT - Food Science and Technology, 51* (1), 375-382.
476 Harnedy, P. A., & FitzGerald, R. J. (2013b). *In vitro* assessment of the
477 cardioprotective, anti-diabetic and antioxidant potential of *Palmaria palmata*
478 protein hydrolysates. *Journal of Applied Phycology, 25* (6), 1793-1803.
479 Harnedy, P. A., O'Keeffe, M. B., & FitzGerald, R. J. (2015). Purification and
480 identification of dipeptidyl peptidase (DPP) IV inhibitory peptides from the
481 macroalga *Palmaria palmata*. *Food Chemistry, 172*, 400-406.
482 Harnedy, P. A., Soler-Vila, A., Edwards, M. D., & FitzGerald, R. J. (2014). The effect
483 of time and origin of harvest on the *in vitro* biological activity of *Palmaria*
484 *palmata* protein hydrolysates. *Food Research International, 62* (0), 746-752.
485 Hernández-Ledesma, B., Dávalos, A., Bartolomé, B., & Amigo, L. (2005).
486 Preparation of antioxidant enzymatic hydrolysates from α-lactalbumin and β-
487 lactoglobulin. Identification of active peptides by HPLC-MS/MS. *Journal of*
488 *Agricultural and Food Chemistry, 53* (3), 588-593.
489 Li, Y. W., & Li, B. (2013). Characterization of structure–antioxidant activity
490 relationship of peptides in free radical systems using QSAR models: Key

sequence positions and their amino acid properties. *Journal of Theoretical Biology, 318* (0), 29-43.
Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy Review, 4* (8), 118-126.
López-Expósito, I., Quirós, A., Amigo, L., & Reci, I. (2007). Casein hydrolysates as a source of antimicrobial, antioxidant and antihypertensive peptides. *Lait, 87*, 241-249.
Nimalaratne, C., Bandara, N., & Wu, J. (2015). Purification and characterization of antioxidant peptides from enzymatically hydrolyzed chicken egg white. *Food Chemistry, 188*, 467-472.
O'Keeffe, M. B., & FitzGerald, R. J. (2015). Identification of short peptide sequences in complex milk protein hydrolysates. *Food Chemistry, 184*, 140-146.
O'Keeffe, M. B., Norris, R., Alashi, M. A., Aluko, R. E., & FitzGerald, R. J. (2017). Peptide identification in a porcine gelatin prolyl endoproteinase hydrolysate with angiotensin converting enzyme (ACE) inhibitory and hypotensive activity. *Journal of Functional Foods, 34*, 77-88.
Power, O., Fernández, A., Norris, R., Riera, F. A., & FitzGerald, R. J. (2014). Selective enrichment of bioactive properties during ultrafiltration of a tryptic digest of β-lactoglobulin. *Journal of Functional Foods, 9* (0), 38-47.
Power, O., Jakeman, P., & FitzGerald, R. J. (2013). Antioxidative peptides: enzymatic production, *in vitro* and *in vivo* antioxidant activity and potential applications of milk-derived antioxidative peptides. *Amino Acids, 44* (3), 797-820.
Sarmadi, B. H., & Ismail, A. (2010). Antioxidative peptides from food proteins: A review. *Peptides, 31* (10), 1949-1956.
Shahidi, F., & Zhong, Y. (2005). Antioxidants: Regulatory Status. In F. Shahidi (Ed.), *Bailey's Industrial Oil and Fat Products* (pp. 491-521): John Wiley & Sons, Inc.
Shen, S., Chahal, B., Majumder, K., You, S. J., & Wu, J. (2010). Identification of novel antioxidative peptides derived from a thermolytic hydrolysate of ovotransferrin by LC-MS/MS. *Journal of Agricultural and Food Chemistry, 58* (13), 7664-7672.
Udenigwe, C. C., & Aluko, R. E. (2011). Chemometric analysis of the amino acid requirements of antioxidant food protein hydrolysates. *International Journal of Molecular Sciences, 12* (5), 3148-3161.
Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology, 39* (1), 44-84.
Walsh, D. J., Bernard, H., Murray, B. A., MacDonald, J., Pentzien, A. K., Wright, G. A., Wal, J. M., Struthers, A. D., Meisel, H., & FitzGerald, R. J. (2004). *In vitro* generation and stability of the lactokinin beta-lactoglobulin fragment (142-148). *Journal of Dairy Science, 87* (11), 3845-3857.
Wang, T., Jónsdóttir, R., Kristinsson, H. G., Hreggvidsson, G. O., Jónsson, J. O., Thorkelsson, G., & Ólafsdóttir, G. (2010). Enzyme-enhanced extraction of antioxidant ingredients from red algae *Palmaria palmata*. *LWT - Food Science and Technology, 43* (9), 1387-1393.
Wang, T., Zhao, Q., & Wang, Q. (2013). Production and antioxidant properties of marine-derived bioactive peptides. In *Marine Proteins and Peptides* (pp. 385-406): John Wiley & Sons, Ltd.

Yuan, Y. V., Bone, D. E., & Carrington, M. F. (2005). Antioxidant activity of dulse (*Palmaria palmata*) extract evaluated *in vitro*. *Food Chemistry, 91* (3), 485-494.

Yuan, Y. V., Carrington, M. F., & Walsh, N. A. (2005). Extracts from dulse (*Palmaria palmata*) are effective antioxidants and inhibitors of cell proliferation *in vitro*. *Food and Chemical Toxicology*, *43* (7), 1073-1081.

Yuan, Y. V., Westcott, N. D., Hu, C., & Kitts, D. D. (2009). Mycosporine-like amino acid composition of the edible red alga, *Palmaria palmata* (dulse) harvested from the west and east coasts of Grand Manan Island, New Brunswick. *Food Chemistry, 112* (2), 321-328.

Zheng, L., Su, G., Ren, J., Gu, L., You, L., & Zhao, M. (2012). Isolation and characterization of an oxygen radical absorbance activity peptide from defatted peanut meal hydrolysate and its antioxidant properties. *Journal of Agricultural and Food Chemistry, 60* (21), 5431-5437.

Zou, T. B., He, T. P., Li, H. B., Tang, H. W., & Xia, E. Q. (2016). The structure-activity relationship of the antioxidant peptides from natural proteins. *Molecules, 21* (1), 72.

Table 1. Oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) values of the *Palmaria palmata* aqueous protein extract, its hydrolysate and solid phase extraction fractions.

Sample	**ORAC value (µmol TE/g dw)**	**FRAP value (µmol TE/g dw)**
Unhydrolysed protein	69.28 ± 3.10 ^{f}	4.31 ± 0.08 ^{e}
Hydrolysate	483.68 ± 31.09 ^{cd}	25.29 ± 0.66 ^{c}
Unbound	241.25 ± 16.39 ^{e}	10.53 ± 0.24 ^{d}
H_2O wash	439.24 ± 31.13 ^{d}	12.66 ± 0.62 ^{d}
10% ACN	853.45 ± 43.01 ^{a}	64.37 ± 4.32 ^{b}
20% ACN	584.40 ± 21.02 ^{bc}	36.73 ± 2.97 ^{c}
25% ACN	1041.73 ± 67.88 ^{a}	64.26± 4.84 ^{b}
30% ACN	971.84 ± 9.08 ^{a}	110.35 ± 2.63 ^{a}
40% ACN	995.81 ± 55.25 ^{a}	137.95 ± 6.98 ^{a}
60% ACN	770.79 ± 53.34 ^{ab}	63.47 ± 4.74 ^{b}

Mean ± SD (n=3). Values with different letters are significantly different at $p<0.05$, TE: Trolox equivalents, dw: dry weight, ACN: Acetonitrile

Table 2. Peptides identified (with flanking N and C terminal residues) in the *Palmaria palmata* aqueous protein extract hydrolysate semi-preparative reverse phase high performance liquid chromatography SP-RP-HPLC-30-F26.

Peptide sequence	**Homologous to:**		**Mass (*m/z*)**	Z	**Mass Error (Da)**
	Rhodophyta species showing 100% homology (accession number)	**Protein**			
K. FITDGNK. R	*Palmaria palmata* [a] (F2ZAL7)	Phycoerythrin beta chain	397.7031	2	0.0021
A. NAATIIK. E	*Porphyra yezoensis (*P59857)	Allophycocyanin beta chain	365.7299	2	-0.0034
A. ANAATIIK. E	*Porphyra yezoensis (*P59857)	Allophycocyanin beta chain	401.2393	2	0.0057
Y.SDITRPGGQM.Y	*Palmaria palmata* [a] (M1VJV1)	Allophycocyanin beta chain	531.2532	2	0.0026
R.DNIQGITKPA.I	*Griffithsia japonica*[b] (Q7XY85)	Histone H4	528.7837	2	0.0041
R. LITGA. A	*Porphyridium purpureum* (P37207)	Phycocyanin alpha chain	474.3021	1	-0.0099
R. LITGAA. Q	*Porphyridium purpureum* (P37207)	Phycocyanin alpha chain	545.3497	1	-0.0204
R. LITGAAQA. V	*Porphyridium purpureum* (P37207)	Phycocyanin alpha chain	372.7164	2	-0.0030
K. LGLSGK. N	*Galdieria sulphuraria* (P23755)	Ribulose bisphosphate carboxylase large chain	287.6778	2	0.0038
R. L/ITL/IAPK. F	*Galdieria sulphuraria*[a] (M2Y2Z5) *Chondrus crispus*[a] (R7QKU4)	Transaldolase	321.7124	2	0.0026
VVPT	Multiple Rhodophyta species[a]	Multiple protein sequences	415.2646	1	-0.0095
QARGAAQA	-	-	772.4042	1	0.0018
FML/IPDAHGL/ITM	-	-	616.8156	2	-0.0219
SDL/ITRPNAGFYT	-	-	671.2998	2	0.0255
L/IL/IASAA	-	-	545.3484	1	-0.0190
L/ISHL/IAGV	-	-	348.7183	2	-0.0128
TAL/IVPR	-	-	328.7056	2	-0.0250

[a] Peptide sequence not evident at protein level, [b]Experimental evidence at transcript level, -: no homology with algal protein sequences

Table 3. Oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) values of synthetic peptides.

Peptide	ORAC value (nmol TE/μmol peptide)	FRAP value (nmol TE/μmol peptide)
FITDGNK	5.79 ± 0.47^{cd}	0.30 ± 0.02^{ef}
NAATIIK	3.70 ± 0.19^{f}	0.16 ± 0.02^{g}
ANAATIIK	9.42 ± 0.60^{b}	0.20 ± 0.02^{fg}
SDITRPGGQM	152.43 ± 2.73^{a}	21.23 ± 0.90^{a}
DNIQGITKPA	5.08 ± 0.12^{de}	0.69 ± 0.06^{e}
LITGA	1.94 ± 0.10^{h}	0.14 ± 0.02^{g}
LITGAA	1.08 ± 0.10^{i}	4.20 ± 0.08^{b}
LITGAAQA	5.91 ± 0.08^{c}	0.57 ± 0.05^{e}
LGLSGK	4.82 ± 0.13^{e}	1.26 ± 0.06^{d}
LTLAPK	nd	0.26 ± 0.05^{fg}
LTIAPK	2.99 ± 0.13^{fg}	0.30 ± 0.01^{ef}
ITLAPK	4.43 ± 0.14^{e}	1.59 ± 0.03^{c}
ITIAPK	1.61 ± 0.08^{hi}	1.17 ± 0.02^{d}
VVPT	0.33 ± 0.08^{j}	0.18 ± 0.01^{g}
QARGAAQA	2.82 ± 0.02^{g}	0.26 ± 0.02^{fg}

Mean ± SD (n=3). Values with different letters are significantly different at $p<0.05$, TE: Trolox equivalents. nd: no activity detected

Table 4.

The effect of simulated gastrointestinal digestion on the peptide stability and antioxidant activity of a *Palmaria palmata* derived synthetic peptide

Sample	ORAC value (nmol TE/µmol peptide)	FRAP value (nmol TE/µmol peptide)	Peptide sequence identified	Mass (*m/z*)	z
SDITRPGGQM	152.43 ± 2.73[a]	21.23 ± 0.90[a]	SDITRPGGQM	531.2557	2
SDITRPGGQM - G	144.60 ± 2.56[a]	21.71 ± 0.52[a]	SDITRPGGQM	531.2571	2
SDITRPGGQM - GI	125.35 ± 2.04[b]	2.18 ± 0.07[b]	SDITRPGGQ	465.7358	2
			SDITRPGG	401.7052	2
			SDIT	435.2076	1
			PGGQ	358.1708	1
			SD	221.0433	1
			QM	278.1857	1
			M	150.0593	1

G: peptide following simulated gastric digestion, GI: peptide following simulated gastrointestinal digestion, TE: Trolox equivalents, nd: not determined. Values represent mean ± SD (n=3). For each peptide and their associated digests values with different letters are significantly different at $p<0.05$

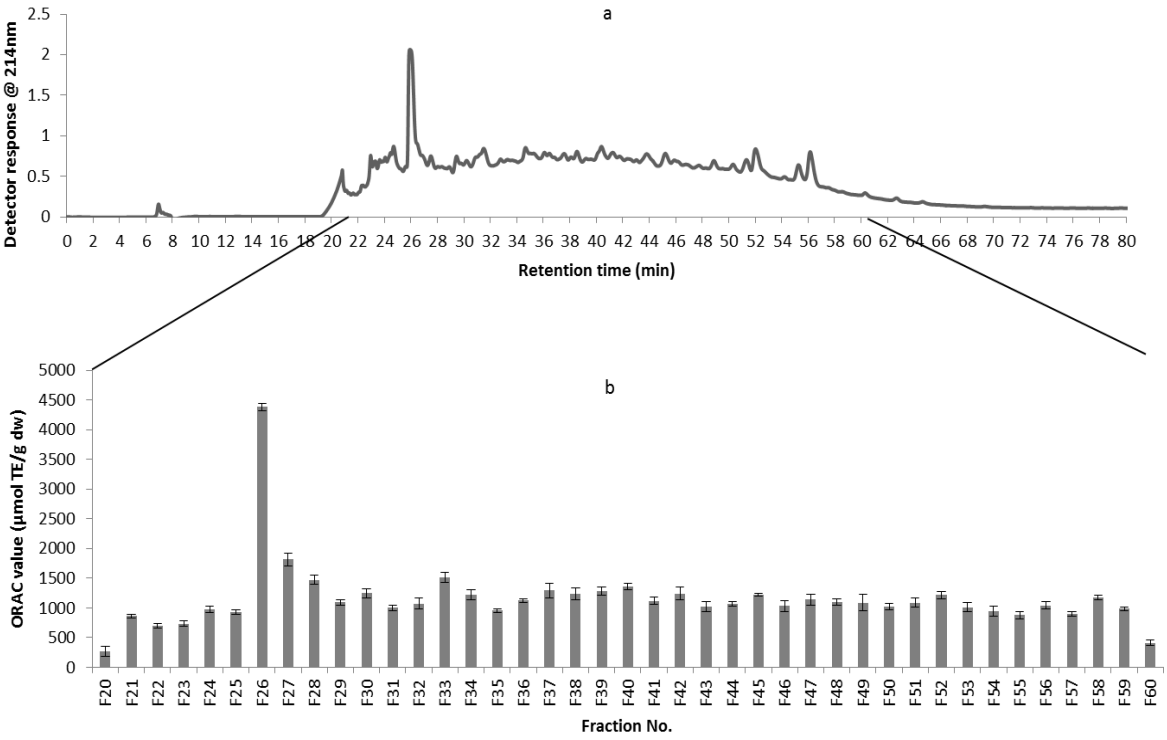

Figure 1. (a) Semi-preparative reverse phase high performance liquid chromatography (SP-RP-HPLC) profile of *Palmaria palmata* aqueous protein hydrolysate solid phase extraction (SPE) fraction SPE-30% and (b) oxygen radical absorbance capacity (ORAC) activity of associated fractions (F20-F60). Mean ± SD (n=3). TE: Trolox equivalents, dw: dry weight.

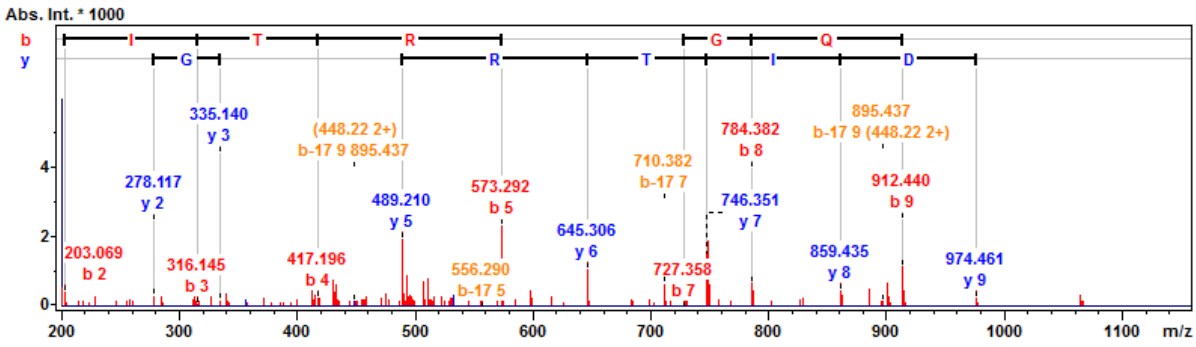

Figure 2. Mass fragmentation spectrum for the *Palmaria palmata* derived peptide SDITRPGGQM (*m*/*z* 531.2532). The *x*-axis represents the *m*/*z* at which the precursor and fragment (*b*, *y*) ions were detected. The *y*-axis shows the relative intensity of ions.